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(54) **A biocompatible, thromboresistant substance comprising hirudin, analogs or fragments thereof, and methods of producing the same.**

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Description

The present invention relates to prosthetic vascular materials, and more specifically to biocompatible, thromboresistant substances and methods of their preparation.

Exposure of blood to artificial surfaces usually leads to deposition of a layer of adherent platelets, accompanied by activation of the intrinsic coagulation system, and ultimately to the formation of a thrombus. In fact, significant blood/materials interaction can occur on a single pass through a prosthetic arterial graft. The types of blood proteins initially adsorbed or bound to synthetic surfaces may include proteins involved in contact coagulation. Contact coagulation or the extrinsic pathway of coagulation is a complex pathway of biochemical events that induces fibrin formation, platelet and complement activation, chemotaxis, kinin generation, and activation of fibrinolytic components. In addition, each of these events augments subsequent biochemical pathways often controlled by positive and negative feedback loops. Thus, thrombosis induced by contact with artificial materials is a major obstacle in the development and use of internal prostheses and extracorporeal devices such as artificial vessels and organs, and cardiopulmonary bypass and hemodialysis equipment.

Materials having varying degrees of thromboresistance have been utilized in vascular prostheses with limited success. These materials include corroding (self-cleaning) metals, synthetic polymers such as polydimethyl siloxane, Teflon, acrylates and methacrylates such as Dacron, electrets*, anionic copolymers, and hydrogels (for a review see Salzman et al. (1987) in Hemostasis and Thrombosis, Basic Principles and Clinical Practice (Colman et al., eds.) J. B. Lippincott Co., Phila. PA, pp. 1335-1347).

To decrease the chances of thrombosis due to extended periods of contact with such artificial materials, patients have been treated with systemically administered anti-coagulant, anti-platelet, and thrombolytic drugs. These include any compound which selectively inhibits thromboxane synthetase without affecting prostacycline synthetase, affects platelet adherence as well as aggregation and release, enhances vascular PGI₂ production, and/or inhibits both thrombin- and thromboxane-mediated platelet aggregation. Such compounds include aspirin, sulfipyrazone, dipyridamole, ticlopidine, and suloctidil. However, treatment with these drugs often elicits unwanted side effects including systemic hemorrhaging and the inability to initiate and complete desired clotting elsewhere in the body.

To improve on the thromboresistance of artificial materials, biologically active molecules having thrombolytic, anticoagulating, thrombogenesis-inhibiting, and/or platelet inhibiting abilities have been linked thereto. For example, heparin has been bound to artificial surfaces to reduce coagulation by activating various inhibitors of the intrinsic clotting system (Salzman et al. (1987) in Hemostasis and Thrombosis: Basic Principles and Clinical Practice, 2nd Ed., (Colman et al., eds.), Lippincott Co., Phila., PA, pp. 1335-1347). However, heparin enhances platelet responses to stimuli such as ADP or collagen, and promotes two adverse primary blood responses towards synthetic surfaces: platelet adhesion and aggregation. In addition, although surface-bound heparin/antithrombin complex may be passive towards platelets, the wide variety of effects it has on interactions with endothelial cell growth factor, inhibition of smooth muscle proliferation, and activation of lipoprotein lipase raises questions as to what adverse effects it may induce over time.

Anti-platelet agents such as pGE₁, pGI₂ (experimental use only), cyclic AMP, and aspirin have also been attached to solid polymer surfaces. These agents discourage the release of platelet factors that stimulate adverse healing responses in the vicinity of a vascular graft. They may also reduce platelet-aided thrombus formation by inhibiting platelet adhesion.

The exposure of many artificial surfaces to albumin prior to vascular contact results in reduced reactivity with platelets (NIH publication No. 85-2185, September, 1985, pp. 19-63). Therefore, albumin has been used to coat extracorporeal surfaces before cardiopulmonary by-pass surgery. However, long-term thromboresistance has not been achieved by this procedure.

EP-A-0,001,853 discloses covalently bonded conjugates of an anticoagulant and a protein; the protein (e.g. albumin) is covalently linked to a substrate and a heparinous anticoagulant is coupled with the protein by use of an amide bond forming agent.

Fibrinolytically active streptokinase and urokinase, alone or in combination with heparin have been attached to artificial surfaces by Kusserow et al (Trans. Am. Soc. Artif. Intern. Organs (1971) 17:1). These enzymes reduce excessive fibrin deposition and/or thrombotic occlusions. However, the long term assessment of their ability to confer thromboresistance to a synthetic surface has not been determined.

Surface active agents such as Pluronic F-68 have also been immobilized on artificial surfaces, but do not appear to offer long term blood compatibility (Salzer et al. (1971) Medical Applications of Plastics, Biomed. Materials Res. Sym. (Gregor, ed.) No. 1 pp. 105).

*electrets are materials that are non-conductors of electricity which exhibit persistent dielectric polarization.

Therefore, what is needed are better biocompatible materials which are thromboresistant in the long term and whose active components do not cause detrimental side affects.

An object of the present invention is to provide a synthetic, biocompatible, thromboresistent material useful for implantable and extracorporeal devices in contact with bodily fluids.

5 Another object is to provide an immobilized thrombogenesis inhibitor which is biologically active, and a method of preparing the same.

Still another object of this invention is to provide a method of inhibiting platelet aggregation, the release of platelet factors, and thrombogenesis at the localized site of the graft or prosthesis-blood interface, thus avoiding the systemic effect of antiplatelet and antithrombosis drugs.

10 Materials and methods are disclosed herein for the provision of biocompatible, thromboresistant substances useful as a component of implantable or extracorporeal devices in contact with the blood.

It has been discovered that a synthetic, biocompatible material can be made into a thromboresistant substance by immobilizing to it, by way of a base coat layer, the thrombogenesis inhibitor hirudin, or an active analog or fragment thereof, in such a way that does not compromise its thrombogenesis inhibiting activity.

15 The term "thrombogenesis inhibitor" is used herein to describe a native, synthetic, or recombinant protein, or fragment thereof having the physical and biochemical characteristics of hirudin.

Synthetic materials contemplated by the instant invention are preferably polymers such as polyethylene terephthalate (e.g. Dacron - TRADE MARK), nylon, polyurethane, cross-linked collagen, poly-
20 tetrafluoroethylene, polyglycolic acid, and mixtures thereof, the most preferred polymeric material being Dacron. Other synthetic materials might also be used.

At least one layer of biocompatible material is adhered to at least one surface of the synthetic material. This base coat layer contains a component which is capable of binding the thrombogenesis inhibitor. Examples of such base coat components include proteins, peptides, lipoproteins, glycoproteins,
25 glycosaminoglycans, hydrogels, synthetic polymers, and mixtures thereof. In preferred aspects of the invention, the base coat layer includes a protein component such as serum albumin or fibronectin from, for example, human or bovine sources, or mixtures of these proteins. Other materials might also be used to form the base coat layer.

In accordance with the invention, the thrombogenesis inhibitor is immobilized on the synthetic material
30 via a base coat layer which is adhered to least one surface of the synthetic material. The base coat layer contains a component capable of binding the thrombogenesis inhibitor without compromising the biological activity of the inhibitor.

In exemplary aspects of the invention, the synthetic material is activated prior to having the base coat layer adhered thereto so as to enhance its ability to bind the base coat base layer. For example, in one preferred aspect, the synthetic material is contacted with a solution which makes available at least one
35 chemically active group (e.g., a carboxylic acid group) in the material for binding to a bifunctional cross-linking reagent (e.g., carbodiimide). The material so treated is then put into contact with a solution containing the cross-linking carbodiimide reagent for a time sufficient to allow the chemically active group to bind thereto.

40 In another embodiment, the synthetic material may be contacted with a solution which removes impurities therein and/or thereon prior to the activation step described above.

The immobilization step may be carried out by initially contacting the thrombogenesis inhibitor with at least one molecule of a bifunctional cross-linking reagent for a time sufficient to allow linking of the reagent to the inhibitor, and then binding the thrombogenesis inhibitor-linked reagent to the base coat. The bound
45 thrombogenesis inhibitor retains its thrombogenesis inhibiting activity when bound to the reagent. The bifunctional cross-linking reagent useful for such an immobilization step may be heterobifunctional (e.g., N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP)), homobifunctional (e.g., ethylene glycolbis (succinimidylsuccinate) (EGS)), or a mixture of both.

The term "bifunctional cross-linking reagent" is defined herein as a molecule having the ability to bind to, and therefore link, two reactive groups on, for example, one molecule or two separate molecules. If the
50 bifunctional cross-linking reagent binds two different types of groups, it is a "heterobifunctional" cross-linking reagent. However, if the bifunctional cross-linking reagent binds only to two similar groups, it is "homobifunctional".

Prior to the binding step, the thrombogenesis-linked reagent may be subjected to chromatographic
55 procedures to remove impurities mixed in with it.

In an alternative aspect of the invention, the base coat adhered to the synthetic material may be linked at the same time to at least one molecule of a bifunctional cross-linking reagent. In this embodiment, the method further includes binding the thrombogenesis inhibitor-linked reagent to the base coat-linked reagent,

thereby linking the thrombogenesis inhibitor to the material-adhered base coat layer.

In another aspect of the invention, the base coat-linked reagent is reduced prior to the binding step. Reduction results in the formation of sulhydryl groups from the reagent on the base coat which can react with the inhibitor-linked bifunctional cross-linking reagent via a substitution reaction to form an S-S bond, thereby covalently linking the thrombogenesis inhibitor to the base coat.

In yet another aspect of the invention, the base coat is linked to the thrombogenesis inhibitor before it is linked to the synthetic, biocompatible material.

The invention will next be described in connection with certain illustrated embodiments. However, it should be clear that various modifications, additions, and deletions can be made without departing from the spirit or scope of the invention.

Brief Description of the Drawing

The foregoing and other objects of the present invention, the various features thereof, as well as the inventions thereof may be more fully understood from the following description when read together with the accompanying drawings in which:

FIGURE 1 is a diagrammatic representation of the pathways involved in thrombogenesis;

FIGURE 2 is a diagrammatic representation of platelet involvement in thrombogenesis; and

FIGURE 3 is a schematic representation of the amino acid sequence of native hirudin.

Description of the Invention

This invention provides biocompatible, thromboresistant substances useful for implantable and extracorporeal devices in contact with the vascular system, and methods for their fabrication.

The substances provided by this invention include a synthetic biocompatible substance having a thrombogenesis-inhibiting reagent linked thereto via a biocompatible base coat adhered to the material's surface.

The material useful in a prosthetic extracorporeal or implantable device may be composed of any biocompatible, synthetic, preferably polymeric material having enough tensile strength to withstand the rigors of blood circulation, and having groups onto which a base coat can be directly or indirectly bound. Examples of such synthetic materials are polytetrafluoroethylene (Teflon - TRADE MARK) and polyethylene terephthalate (e.g. Dacron), nylon and the like. The material may have any dimensions suitable for the purpose for which it is being used. For example, it may be an integral part of an implanted heart valve or of an extracorporeal device used for hemodialysis or cardiopulmonary by-pass surgery, or it may be used to coat catheters or to line the interior of a vascular graft.

The synthetic material, when obtained, may be coated with or contain various noncovalently adhered impurities whose removal may be prerequisite for the adherence of a base coat thereto. For example, lubricants on commercial quality Dacron can be removed by contacting the Dacron with a solution containing, for example, various detergents, solvents, or salts, which loosen and/or solubilize these impurities.

TABLES 1 and 2 outline representative methods of preparing the biocompatible, thromboresistant substance, where "Da" refers to a synthetic material composed of woven Dacron fibers, and "HSA" refers to human serum albumin. The abbreviation EDC refers to carbodiimide, which is obtainable from the Pierce Chemical Company.

TABLE 1

STEP	PROCESS
1)	Da. + NaOH \longrightarrow Da-COOH
2)	Da-COOH + EDC \longrightarrow Da-EDC
3)	Da-EDC + HSA \longrightarrow Da-HSA + urea (EDC by-product)
4)	Da-HSA + SPDP \longrightarrow Da-HSA-SPDP
5)	Da-HSA-SPDP + DTT \longrightarrow Da-HSA-SH + P-2-T
6)	Inhibitor + SPDP \longrightarrow Inhibitor-SPDP
7)	Da-HSA-SH + Inhibitor-SPDP \longrightarrow Da-HSA-S-S-Inhibitor + P-2-T

TABLE 2

STEP	PROCESS
1)	HSA + SPDP \longrightarrow HSA-SPDP
2)	HSA-SPDP + DTT \longrightarrow HSA-SH + P-2-T
3)	Inhibitor + SPDP \longrightarrow Inhibitor-SPDP
4)	HSA-SH + Inhibitor-SPDP \longrightarrow HSA-S-S-Inhibitor + P-2-T
5)	Da + NaOH \longrightarrow Da-COOH
6)	Da-COOH + EDC \longrightarrow Da-EDC
7)	Da-EDC + HSA-S-S-Inhibitor \longrightarrow Da-HSA-S-S-Inhibitor + urea (EDC by-product)

Initially, the material may be activated so as to enhance the binding of the base coat layer. This activating step increases the number of chemically active groups in the material. For example, alkaline hydrolysis may be performed to increase the number of reactive carboxylic acid groups in the Dacron to which a bifunctional cross-linking reagent such as carbodiimide may be bound. Ultimately, the base coat will adhere to the bound carbodiimide groups on the material. However, this method must be performed with care, as alkaline hydrolysis partially degrades the Dacron, resulting in a fraying of the material's fibers.

At least one base coat layer is adhered to at least one surface of the synthetic material.

This layer, either adhered to the material or unbound, provides components for attachment of the thrombogenesis inhibitor. Such components provide more binding sites for the inhibitor than the synthetic material, alone, thereby amplifying the amount of inhibitor which may be bound. Useful components include proteins, peptides, lipoproteins, glycoproteins, glycosaminoglycans, synthetic polymers, and mixtures thereof. Proteins such as serum albumin and fibronectin are particularly useful for this purpose as they are known to have anti-thrombogenic properties, themselves, are very desirable as base coat components (Lyman et al. (1965) Trans. Am. Soc. Artif. Intern. Organs 11:301; Falb et al. (1971) Fed. Proc. 30:1688). An HSA molecule, for example, has 65 amino groups available as binding sites.

Attachment of the base coat to the artificial surface may be covalent in nature.

Methods to covalently bind proteins to Dacron involve attack of the free reactive succinimide ester group of the cross-linking reagent to primary amino groups on a protein. As shown in the example in TABLE 1, to covalently adhere the base coat to Dacron, the Dacron is initially treated with 0.5 N NaOH and reacted with carbodiimide before it is coated with HSA (base coat) in phosphate buffered saline (PBS).

A thrombogenesis inhibitor useful as a coating for surfaces in contact with blood, bodily fluids, or tissues, is then covalently adhered to the base coat via the component. Inhibitor-coated substances are ideal for implantable use in devices which are in direct contact with blood. For example, by-pass grafts used to replace blood vessels often become filled with blood clots or thrombi, resulting in restricted blood flow. Since the inhibitor-coated substance is resistant to formation of blood clots, thrombosis and subsequent blockage of the bypass graft will be prevented. Likewise when catheters are placed into the vascular system for a diagnostic or therapeutic purposes, a blood clot often forms on the outside of the catheter. The clot may be washed off the catheter by flowing blood, or be jarred loose by manipulation of the catheter, increasing the possibility of embolism and blockage of the circulation to vital organs. Inhibitor-coated substances provide similar advantages for artificial or prosthetic heart valves, intraaortic balloon pumps, total or artificial heart or heart assist devices, intracaval devices, and any device in contact with the bloodstream. In addition, inhibitor-coated devices provide advantages for intracavity devices such as intraperitoneal dialysis catheters and subcutaneous implants where the thrombogenesis-induced inflammatory reactions would be diminished.

Thrombogenesis inhibitors useful for these purposes include hirudin and active analogs, fragments, derivatives, and fusion products thereof, or mixtures thereof.

Hirudin is a protein isolated from the saliva of leeches, the amino acid sequence of which is shown in FIG. 3. Hirudin has been shown to reduce platelet adhesiveness, a characteristic which is probably attributed to its mode of action on thrombin. This property alone makes hirudin a most attractive anticoagulant when synthetic surfaces interface with blood. It also selectively inhibits the ability of thrombin to be proteolytic, to be mitogenic for fibroblasts, to activate platelets, and to have chemotatic properties for monocytes and polymorphonuclear leukocytes. In addition, the immobilized hirudin-thrombin complex may down-regulate the events of thrombin mediated chemotaxis. This is a significant event as chronic inflammation may be due to the release by polymorphonuclear leukocytes of degradative enzymes and superoxides throughout the graft that show effects at the sites of the anastomosis, contributing to anastomotic hyperplasia.

A number of synthetic and recombinant hirudin analogs exist (e.g., CGP39393 produced by recombinant DNA techniques by Ciba-Geigy, Basel, Switzerland; PCT WO79/00638; PCT WO86/03517) which are at least equally useful as thrombogenesis inhibitors.

5 The thrombogenesis inhibitor is directly or indirectly immobilized on the base coat via the use of a bifunctional cross-linking reagent. In particular, a heterobifunctional cross-linking reagent which has two different reactive groups at each end of a linear molecule, and can therefore bind two different reactive groups on other molecules or on a different region of the same molecule, is most useful as a bifunctional cross-linking agent. For example, photoreactive cross-linkers, such as sulfosuccinimidyl 2-(m-azido-o-nitrobenzamido)ethyl-1, 3'-dithio-propionate (SAND), or N-succinimidyl-6-(4- azido-2'-nitrophenyl-amino) hex-
10 anoate (SANPAH) have a photoreactive group that can directly insert into C-H bonds of the base coat by photochemical coupling, while the other group remains free to bind to proteins.

Other useful and preferable cross-linking reagents (such as SPDP) and their characteristics are found in TABLE 3. In TABLE 3, the "Double-Agent Number" listed for each reagent is the commercial designation for the reagent as made available by pierce Chemical Co. (Rockford, Illinois).

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TABLE 3 (Part A)

CROSS-LINKING REAGENTS

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10	Double- Agent Number	Double- Agent Acronym	Bifunctionality		Reactive towards:		
			Homo	Hetero	NH ₂	SH	Photo- Reactive
15	21551	ANB-NOS		X	X		X
	20106	APB		X		X	X
	20107	APG		X			X
20	21559	APTP		X		X	X
	21579	BS ³	X		X		
	22319	BMH	X			X	
	21554	BSOCOES	X		X		
25	21524	DFDNB	X		X		
	20047	DIDS	X		X		
	20664	DMA	X		X		
30	20666	DMP	X		X		
	20668	DMS	X		X		
	22585	DSP	X		X		
35	21555	DSS	X		X		
	20590	DST	X		X		
	20665	DTBP	X		X		
	22590	DTBPA	X				X
40	21577	DTSSP	X		X		
	21550	EADB		X	X		X
	21565	EGS	X		X		
45	23700	FNPA		X	X		X
	21560	HSAB		X	X		X

50

55

TABLE 3 (Cont'd)

CROSS-LINKING REAGENTS

5

	Double- Agent Number	Double- Agent Acronym	Bifunctionality		Reactive towards:		
			Homo	Hetero	NH ₂	SH	Photo- Reactive
10							
15	26095	MABI		X	X		X
	22310	MBS		X	X	X	
	27715	NHS-ASA		X	X		X
	20669	PNP-DTP		X	X		X
20	21552	SADP		X	X		X
	21549	SAND		X	X		X
	22588	SANPAH		X	X		X
25	27716	SASD		X	X		X
	22325	SIAB		X	X	X	X
	22320	SMCC		X	X	X	
30	22315	SMPB		X	X	X	
	21557	SPDP		X	X	X	
	21556	Sulfo-					
35		BSOCOES	X		X		
	20591	Sulfo-					
		DST	X		X		
	21556	Sulfo-					
40		EGS	X		X		
	22312	Sulfo-					
		MBS		X	X	X	
45	21553	Sulfo-					
		SADP		X	X		X
	22539	Sulfo-					
50		SANPAH		X	X		X

55

TABLE 1 (Cont'd)

CROSS-LINKING REAGENTS

Double- Agent Number	Double- Agent Acronym	Bifunctionality Homo Hetero	Reactive towards:		
			SH ₂	SH	Photo- Reactive
22327	Sulfo- SIAB	X	X	X	
22322	Sulfo- SMCC	X	X	X	
22317	Sulfo- SMPB	X	X	X	
26101	TRAUT'S	X	X		

TABLE 1 (Cont'd)

CROSS-LINKING REAGENTS (part B)

<u>Agent Acronym</u>	<u>Chemical Name</u>
ANB-HOS	N-5-azido-2-nitrobenzoyloxysuccinimide
APB	p-azidophenacyl bromide
APG	p-azidophenyl glyoxal
APTP	n-4-(azidophenylthio)phthalimide
BS ³	bis(sulfosuccinimidyl) suberate
BMH	bis maleimidohexane
BSOCOES	bis[2-(succinimidooxycarbonyloxy)- ethyl]sulfone
DFDNB	1,5-difluoro-2,4-dinitrobenzene
DIDS	4,4'-diisothiocyano-2,2'-disulfonic acid stilbene

TABLE 1 (Cont'd)

5	<u>Agent Acronym</u>	<u>Chemical Name</u>
	DMA	dimethyl adipimidate-2 HCl
	DMP	dimethyl pimelimidate-2 HCl
10	DMS	dimethyl suberimidate-2 HCl
	DSP	dithiobis(succinimidylpropionate)
15	DSS	disuccinimidyl suberate
	DST	disuccinimidyl tartarate
	DTBP	dimethyl 3,3'-dithiobispropionimidate-2-HCl
20	DTBPA	4,4'-dithiobisphenylazide
	DTSSP	3,3'-dithiobis(sulfosuccinimidylpropionate)
25	EADB	ethyl-4-azidophenyl 1,4-dithiobutyrimidate
30	EGS	ethylene glycolbis(succinimidylsuccinate)
	FNPA	1-azido-4-fluoro-3-nitobenzene
35	HSAB	N-hydroxysuccinimidyl-4-azidobenzoate
	MABI	methyl-4-azidobenzoimidate
	MBS	m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester
40	NHS-ASA	N-hydroxysuccinimidyl-4-azidosalicylic acid
45	PNP-DTP	p-nitrophenyl-2-diazo-3,3,3-trifluoropropionate
	SADP	N-succinimidyl(4-azidophenyl)-1,3'-dithiopropionate
50	SAND	sulfosuccinimidyl 2-(m-azido-o-nitrobenzamido)-ethyl-1,3'-dithiopropionate

TABLE 3 (Cont'd)

5	<u>Agent Acronym</u>	<u>Chemical Name</u>
	SANPAH	N-succinimidyl-6-(4'-azido-2'-nitro-phenyl-amino)hexanoate
10	SASD	sulfosuccinimidyl 2-(p-azidosalicyl-amido)ethyl-1,3'-dithio-propionate
	SIAB	N-succinimidyl(4-iodoacetyl)amino-benzoate
15	SMCC	succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate
	SMPB	succinimidyl 4-(p-maleimidophenyl)-butyrate
20	SPDP	N-succinimidyl 3-(2-pyridyldithio)propionate
25	Sulfo-BSOCSS	bis(2-(sulfosuccinimidoxy-carbonyl-oxy)ethyl)sulfone
	Sulfo-DST	disulfosuccinimidyl tartarate
30	Sulfo-EGS	ethylene glycolbis(sulfosuccinimidyl-succinate)
	Sulfo-MBS	m-maleimidobenzoyl-N-hydroxysulfo-succinimide ester
35	Sulfo-BADP	sulfosuccinimidyl(4-azidophenyldithio)-propionate
40	Sulfo-SANPAH	sulfosuccinimidyl 6-(4'azido-2'-nitrophenylamino)hexanoate
	Sulfo-SIAB	sulfosuccinimidyl(4-iodoacetyl)amino-benzoate
45	Sulfo-SMCC	sulfosuccinimidyl 4-(N-maleimido-methyl)cyclohexane-1-carboxylate
	Sulfo-SMPB	sulfosuccinimidyl 4-(p-maleimido-phenyl)butyrate
50	TRAUT'S	2-iminothiolane-HCl

55 The cross-linking reagent is applied to the base coat in amounts such that the desired binding site density is achieved. Binding site density is that amount of cross-linking reagent, in terms of moles/g synthetic material, to bind to the base coat while providing confluent coverage of the surface.

To put the inhibitor in condition for linkage to the base coat, the cross-linking reagent may be initially coupled separately to both the base coat and to the inhibitor. The kinetic constants of the inhibitors are

compared before and after coupling to evaluate effects of the procedure on their kinetic constants. The inhibitor should remain biologically active after being coupled. Therefore, standard activity assays specific for the inhibitor to be immobilized are performed using a standard thrombin solution to evaluate this capacity.

5 As an alternative, the protein component of the base coat may be bound to the thrombogenesis inhibitor forming a conjugate prior to its adherence to the synthetic material, and the conjugate bound to the synthetic material as shown in TABLE 2. In addition, thrombogenesis inhibitor conjugate retains biological activity, and can be used as an agent to increase the half life in the circulation as it is not easily cleared by the kidney.

10 In the special case of SPDP derivatization of hirudin, linkage of certain groups on hirudin to SPDP may destroy hirudin's biological activity because at least some of these groups are required for activity. However, by adjusting the reaction ratio of hirudin to SPDP (1:4, mole:mole), and running the reaction at near physiological pH, SPDP becomes somewhat selective for epsilon amino groups. The result of these conditions favor a 1:1 (mole:mole) conjugation ratio of hirudin to SPDP covalently bound without destroying
15 hirudin's biological activity.

SPDP will react with terminal as well as epsilon amino groups. Since derivatization of a terminal amino group can inactivate a biologically active protein, t-butyl-dicarbonate (T-BLOCK (Pierce Chemical Co., Rockford, Illinois)) may be used to block that group during SPDP-derivatization. The blocking group is then removed after derivatization to restore biological activity.

20 The invention will be further understood from the following, non-limiting examples.

EXAMPLE 1

A. Pretreatment and Activation of Dacron

25 Dacron polyester 52 (DuPont) is sectioned into 1.0 cm lengths. The lubricant on and in the woven surface is removed by washing once for 1 hr with carbon tetrachloride, and twice with 100% CH₃OH. The methanol is removed by multiple water washes, followed by one wash in phosphate buffered saline (PBS), pH 7.4.

30 The graft material is then subjected to alkaline hydrolysis to increase available COOH groups. The material is treated with 0.5 M NaOH at 50°C for 1 hr. It is then washed with H₂O repeatedly, and the following steps initiated immediately.

B. Carbodiimide Derivatization of Activated Dacron

35 The activated material is placed into 100.0 ml of 10 mM water-soluble carbodiimide (EDC) in deionized water, pH 4.6-5.0, for 1 hour at RT with constant stirring. The material is removed and washed in PBS to remove excess unbound EDC.

C. Base Coat Formation

40 The base coat is applied to the lumen of the Dacron graft material. The derivatized Dacron material is incubated in a 5% HSA solution in PBS at 1 ml/cm² graft material for 24 hr at RT with constant stirring. The graft is removed and washed in PBS to remove nonspecifically bound HSA. Approximately 20 mg
45 protein/mg Dacron is covalently bound.

D. Linkage of SPDP to the Base Coat

50 The HSA-bound Dacron material is incubated in a 1.0 mM solution of SPDP in PBS, pH 7.4, to bind SPDP to the HSA (100 mM SPDP/cm² base coat). Incubation is terminated after 30-40 min at RT. The graft is washed in PBS to remove nonspecifically bound SPDP.

E. Activation of SPDP on Base Coat and Measurement of Binding Site Density

55 The SPDP-linked material is dried and weighed to obtain its absolute weight. It is then placed in a 50 mM solution of dithiothreitol (DTT) for 5 min at RT. This reaction releases pyridine-2-thione (P-2-T) from the bound SPDP, and simultaneously forms free sulfhydryl (SH) groups on the base coat. The released P-2-T is quantitated by absorption spectrophotometry at 343 nm using its extinction coefficient ($E = 8.08 \times 10^3$),

and is directly proportional to the quantity of bound SPDP or binding sites. The number of binding sites are calculated and expressed as moles of sites/g of Dacron.

The material is then washed 5 times in PBS and 4 times in distilled water.

5 F. Linkage of SPDP to Hirudin

Lyophilized hirudin is resuspended in PBS at 1 mg/ml. SPDP (Pharmacia, Piscataway, NJ) is dissolved in 100% ETOH to 10 mM. One part hirudin is mixed with 4 parts SPDP (mole:mole), and incubated for 30 min at RT. SPDP-bound-hirudin is then separated from free SPDP and reaction by-products by chromatog-
10 raphy on a G-25 column; the derivatized hirudin is eluted first.

G. Measurement of SPDP Bound to Hirudin

The binding of SPDP to hirudin can be quantitated by the addition of DTT which liberates pyridine-2-
15 thione (P-2-T) from SPDP bound to hirudin, and which can be measured spectrophotometrically at 343 nm. From this measurement, the moles of SPDP bound to hirudin can be calculated. Each P-2-T released represents one covalent attachment of SPDP to hirudin. One mole of hirudin binds per 1.2 moles SPDP in the present studies.

20 H. Linkage of Derivatized Hirudin to Base Coat

The reduced SPDP-linked base coat (having free SH groups) is washed with PBS to remove the DTT. SPDP-linked hirudin is then added to the graft at 50.0 mg/cm² Dacron. The solution is incubated overnight at RT to allow the binding of SPDP-hirudin to SH groups on the Dacron graft. The Dacron material with
25 hirudin covalently immobilized thereto is then washed and stored in PBS.

I. Analysis

A. Spectrophotometric Assays:

To quantitate the amount of SPDP-hirudin immobilized on the base coat, an absorbance reading is
30 taken at 343 nm of the solution at the time of the addition of SPDP-hirudin to the Dacron. After the overnight incubation period, another absorbance reading is taken, and the change in absorbance is due to the quantity of P-2-T released from SPDP-hirudin. The amount of P-2-T released is directly proportional to the number of SPDP substitution reactions that have occurred between the base coat SH
35 groups and SPDP-hirudin.

B. Thrombin Inhibition Assay:

Using a known amount of thrombin, a standard curve is constructed with known amount of hirudin or
40 hirudin analog by adding hirudin to thrombin, and measuring residual thrombin activity using chromatographic substrate S-2238. Thrombin inhibition by derivatized hirudin (hirudin-SPDP) or immobilized hirudin (the Dacron material with immobilized hirudin) is then compared to nonderivatized hirudin values by equating ED50 values using 10 NIH units/ml thrombin, and measuring residual thrombin activity.

The invention may be embodied in other specific forms without departing from the spirit or essential
characteristics thereof. The present embodiments are therefore to be considered in all respects as
45 illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

Claims

Claims for the following Contracting States : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

50

1. A biocompatible, thromboresistant substance comprising:

- (a) a synthetic, biocompatible material;
- (b) at least one biocompatible base coat layer adhered to at least one surface of said material; and
- (c) a thrombogenesis inhibitor immobilized on said base coat layer through the use of a bifunctional
55 cross-linking reagent, for example a heterobifunctional or homobifunctional cross-linking reagent, said inhibitor being hirudin or an active analog or fragment thereof,
said base coat layer having a component capable of binding said thrombogenesis inhibitor, and
said inhibitor having thrombogenesis inhibiting activity when immobilized.

2. The substance of claim 1, wherein said material comprises a polymer, for example a polymer selected from the group consisting of polyethylene terephthalate, (e.g. Dacron), nylon, polyurethane, cross-linked collagen, polyglycolic acid, polytetrafluoroethylene, and mixtures thereof.
- 5 3. The substance of claim 1 or claim 2, wherein said base coat layer comprises a component selected from the group consisting of a protein, peptide, lipoprotein, glycoprotein, glycosaminoglycan, hydrogel, synthetic polymer and mixtures thereof.
- 10 4. The substance of claim 3, wherein said component of said base coat layer comprises a protein; for example a protein selected from the group consisting of serum albumin (e.g. bovine serum albumin and human serum albumin), fibronectin (e.g. bovine fibronectin and human fibronectin), and mixtures thereof.
- 15 5. The substance of claim 1 wherein the bifunctional cross-linking reagent is SPDP.
6. A method of producing a biocompatible, thromboresistant substance, said method comprising the steps of:
 - (a) adhering at least one base coat layer to at least one surface of a synthetic, biocompatible material, said base coat layer containing a component capable of binding a thrombogenesis inhibitor; and
 - 20 (b) immobilizing said thrombogenesis inhibitor to said base coat layer through the use of a bifunctional cross-linking reagent, for example a heterobifunctional or homobifunctional cross-linking reagent, said inhibitor being hirudin or an active analog or fragment thereof, and having thrombogenesis inhibiting activity when immobilized.
- 25 7. The method of claim 6, wherein said adhering step comprises:
 - (a) activating said material so as to enhance the binding of said base coat layer thereto; and
 - (b) contacting said activated material with said base coat layer for a time sufficient to allow said component of said base coat layer to bind to said activated material.
- 30 8. The method of claim 6, wherein said adhering step comprises adhering a base coat layer to at least one surface of said material, said base coat layer containing a component selected from the group consisting of a protein, peptide, lipoprotein, glycoprotein, hydrogel, glycosaminoglycan, synthetic polymer, and mixtures thereof.
- 35 9. The method of claim 8, wherein said adhering step further comprises adhering to at least one surface of said material a base coat layer containing a protein, for example a protein selected from the group consisting of serum albumin (for example human serum albumin and bovine serum albumin), fibronectin (for example human fibronectin and bovine fibronectin), and mixtures thereof.
- 40 10. The method of claim 7, wherein said activating step comprises the steps of:
 - (a) treating said material with a solution that makes available for binding at least one chemically reactive group in said material; and
 - 45 (b) contacting said treated material with a solution containing a bifunctional cross-linking reagent for a time sufficient to allow binding of said chemically reactive group to said reagent.
- 50 11. The method of claim 10, wherein said treating step further comprises treating said material with a solution that makes available for binding at least one chemically active group in said material, said chemically active group being a carboxylic acid group.
12. The method of claim 6 further comprising the preliminary step of contacting said material with a solution which removes impurities thereon, said preliminary step being performed prior to said adhering step.
- 55 13. The method of claim 6, wherein said immobilizing step further comprises the steps of:
 - (a) contacting said thrombogenesis inhibitor with at least one molecule of a bifunctional cross-linking reagent for a time sufficient to allow linking of said reagent to said thrombogenesis inhibitor; and

(b) binding said thrombogenesis inhibitor-linked reagent to said base coat, said linked thrombogenesis inhibitor having thrombogenesis inhibiting activity.

5 14. The method of claim 13, wherein said contacting step further comprises contacting said base coat with at least one molecule of said bifunctional cross-linking reagent for a time sufficient to allow linking of said reagent to said base coat,
and said binding step further includes binding said thrombogenesis inhibitor-linked reagent to said base coat-linked reagent.

10 15. The method of claim 14 further comprising the steps of:
(a) reducing said base coat-linked reagent to expose a sulfhydryl group thereon;
(b) adding said inhibitor-linked reagent to the exposed sulfhydryl group thereon; and
(c) inducing a substitution reaction involving said sulfhydryl group and said inhibitor-linked reagent, said reaction resulting in linkage of said base coat to said inhibitor.

15 16. The method of any of claims 13, 14 or 15, wherein said bifunctional cross-linking reagent is selected from the group consisting of heterobifunctional cross-linking reagents (for example SPDP), homobifunctional cross-linking reagents, and mixtures thereof.

20 17. The method of claim 13 further comprising the additional step of subjecting said thrombogenesis-linked reagent to a chromatographic procedure to remove impurities therein, said additional step being performed after said contacting step and prior to said binding step.

25 18. A method of producing a biocompatible, thromboresistant substance, said method comprising the steps of:

(a) immobilizing a thrombogenesis inhibitor to a base coat layer through the use of a bifunctional cross-linking reagent, for example a heterobifunctional or homobifunctional cross-linking reagent, said inhibitor being hirudin or an active analog or fragment thereof, and having thrombogenesis inhibiting activity when immobilized, and
30 said base coat layer containing a component capable of binding said thrombogenesis inhibitor; and
(b) adhering said base coat layer linked to said thrombogenesis inhibitor to at least one surface of a synthetic, biocompatible material.

35 **Claims for the following Contracting States : ES, GR**

1. A method of producing a biocompatible, thromboresistant substance, said method comprising the steps of:

40 (a) adhering at least one base coat layer to at least one surface of a synthetic, biocompatible material, said base coat layer containing a component capable of binding a thrombogenesis inhibitor; and
(b) immobilizing said thrombogenesis inhibitor to said base coat layer through the use of a bifunctional cross-linking reagent, for example a heterobifunctional or homobifunctional cross-linking reagent, said inhibitor being hirudin or an active analog or fragment thereof, and having thrombogenesis inhibiting activity when immobilized.

2. The method of claim 1, wherein said adhering step comprises:

50 (a) activating said material so as to enhance the binding of said base coat layer thereto; and
(b) contacting said activated material with said base coat layer for a time sufficient to allow said component of said base coat layer to bind to said activated material.

3. The method of claim 2, wherein said activating step comprises the steps of:

55 (a) treating said material with a solution that makes available for binding at least one chemically reactive group in said material; and
(b) contacting said treated material with a solution containing a bifunctional cross-linking reagent for a time sufficient to allow binding of said chemically reactive group to said reagent.

4. The method of claim 3, wherein said treating step further comprises treating said material with a solution that makes available for binding at least one chemically active group in said material, said chemically active group being a carboxylic acid group.
5. The method of claim 1 further comprising the preliminary step of contacting said material with a solution which removes impurities thereon, said preliminary step being performed prior to said adhering step.
6. The method of claim 1, wherein said immobilizing step further comprises the steps of:
 - (a) contacting said thrombogenesis inhibitor with at least one molecule of a bifunctional cross-linking reagent for a time sufficient to allow linking of said reagent to said thrombogenesis inhibitor; and
 - (b) binding said thrombogenesis inhibitor-linked reagent to said base coat, said linked thrombogenesis inhibitor having thrombogenesis inhibiting activity.
7. The method of claim 6, wherein said contacting step further comprises contacting said base coat with at least one molecule of said bifunctional cross-linking reagent for a time sufficient to allow linking of said reagent to said base coat, and said binding step further includes binding said thrombogenesis inhibitor-linked reagent to said base coat-linked reagent.
8. The method of claim 7 further comprising the steps of:
 - (a) reducing said base coat-linked reagent to expose a sulfhydryl group thereon;
 - (b) adding said inhibitor-linked reagent to the exposed sulfhydryl group thereon; and
 - (c) inducing a substitution reaction involving said sulfhydryl group and said inhibitor-linked reagent, said reaction resulting in linkage of said base coat to said inhibitor.
9. The method of any of claims 6, 7 or 8, wherein said bifunctional cross-linking reagent is selected from the group consisting of heterobifunctional cross-linking reagents (for example SPDP), homobifunctional cross-linking reagents, and mixtures thereof.
10. The method of claim 6 further comprising the additional step of subjecting said thrombogenesis-linked reagent to a chromatographic procedure to remove impurities therein, said additional step being performed after said contacting step and prior to said binding step.
11. A method of producing a biocompatible, thromboresistant substance, said method comprising the steps of:
 - (a) immobilizing a thrombogenesis inhibitor to a base coat layer through the use of a bifunctional cross-linking reagent, for example a heterobifunctional or homobifunctional cross-linking reagent, said inhibitor being hirudin or an active analog or fragment thereof, and having thrombogenesis inhibiting activity when immobilized, and said base coat layer containing a component capable of binding said thrombogenesis inhibitor; and
 - (b) adhering said base coat layer linked to said thrombogenesis inhibitor to at least one surface of a synthetic, biocompatible material.
12. The method of any of claims 1 to 11, wherein said material comprises a polymer, for example a polymer selected from the group consisting of polyethylene terephthalate (e.g. Dacron), nylon, polyurethane, cross-linked collagen, polyglycolic acid, polytetrafluoroethylene, and mixtures thereof.
13. The method of any of claims 1 to 12, wherein said base coat layer comprises a component selected from the group consisting of a protein, peptide, lipoprotein, glycoprotein, glycosaminoglycan, hydrogel, synthetic polymer and mixtures thereof.
14. The method of claim 13, wherein said component of said base coat layer comprises a protein; for example a protein selected from the group consisting of serum albumin (e.g. bovine serum albumin and human serum albumin), fibronectin (e.g. bovine fibronectin and human fibronectin), and mixtures thereof.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Bioverträgliche, thromboresistente Substanz, bestehend aus oder enthaltend:
 - (a) einem synthetischen, bioverträglichen Material,
 - (b) mindestens einer bioverträglichen Grundschrift, die auf mindestens einer Fläche dieses Materials fest haftet und
 - (c) einem Thrombeninhibitor, der durch Verwendung eines bifunktionellen Vernetzungsreagens, z. B. eines heterobifunktionellen oder homobifunktionellen Vernetzungsreagens, auf der Grundschrift immobilisiert ist, wobeider Inhibitor Hirudin oder ein aktives Analog oder Fragment davon ist, die Grundschrift eine Komponente enthält, die in der Lage ist, den Thrombeninhibitor zu binden, und der Inhibitor die Thrombenbildung inhibiert, wenn er immobilisiert ist.
2. Substanz gemäß Anspruch 1, dadurch gekennzeichnet, daß das Material ein Polymer enthält oder daraus besteht, z. B. ein Polymer aus der Gruppe bestehend aus Polyethylenterephthalat (z. B. Dacron), Nylon, Polyurethan, vernetztem Collagen, Polyglykolsäure, Polytetrafluorethylen sowie deren Gemischen.
3. Substanz gemäß Anspruch 1 oder Anspruch 2, dadurch gekennzeichnet, daß die Grundschrift eine Komponente enthält, die aus der Gruppe bestehend aus Protein, Peptid, Lipoprotein, Glykoprotein, Glycosaminoglycan, Hydrogel, synthetischem Polder sowie deren Gemischen ausgewählt ist.
4. Substanz gemäß Anspruch 3, dadurch gekennzeichnet, daß die Komponente der Grundschrift ein Protein enthält oder daraus besteht, z. B. ein Protein aus der Gruppe bestehend aus Serumalbumin (z. B. Rinderserumalbumin und Humanserumalbumin), Fibronectin (z. B. Rinderfibronectin und Humanfibronectin) sowie deren Gemischen.
5. Substanz gemäß Anspruch 1, dadurch gekennzeichnet, daß das bifunktionelle Vernetzungsreagens SPDP ist.
6. Verfahren zur Herstellung einer bioverträglichen, thromboresistenten Substanz, das aus folgenden Schritten besteht:
 - (a) Anbringen von mindestens einer Grundschrift auf mindestens einer Fläche eines synthetischen, bioverträglichen Materials, wobei die Grundschrift eine Komponente enthält, die in der Lage ist, einen Thrombeninhibitor zu binden, und
 - (b) Immobilisierung des Thrombeninhibitors auf der Grundschrift durch Verwendung eines bifunktionellen Vernetzungsreagens, z. B. eines heterobifunktionellen oder homobifunktionellen Vernetzungsreagens, wobei der Inhibitor Hirudin oder ein aktives Analog oder Fragment davon ist und die Thrombenbildung inhibiert, wenn er immobilisiert ist.
7. Verfahren gemäß Anspruch 6, dadurch gekennzeichnet, daß das Anbringen folgende Schritte umfaßt:
 - (a) Aktivierung des Materials, um die Bindung der Grundschrift auf dem Material zu erhöhen, und
 - (b) Kontaktierung des aktivierten Materials mit der Grundschrift über einen ausreichend langen Zeitraum, um die Komponente der Grundschrift auf dem aktivierten Material zu binden.
8. Verfahren gemäß Anspruch 6, dadurch gekennzeichnet, daß das Anbringen darin besteht, eine Grundschrift auf mindestens einer Fläche des Materials fest anzubringen, wobei die Grundschrift eine Komponente enthält, die aus der Gruppe bestehend aus Protein, Peptid, Lipoprotein, Glykoprotein, Glycosaminoglycan, Hydrogel, synthetischem Polymer sowie deren Gemischen ausgewählt ist.
9. Verfahren gemäß Anspruch 8, dadurch gekennzeichnet, daß das Anbringen außerdem darin besteht, auf mindestens einer Fläche des Materials eine Grundschrift fest anzubringen, die ein Protein enthält, z. B. ein Protein aus der Gruppe bestehend aus Serumalbumin (z. B. Humanserumalbumin und Rinderserumalbumin), Fibronectin (z. B. Humanfibronectin und Rinderfibronectin) sowie deren Gemischen.
10. Verfahren gemäß Anspruch 7, dadurch gekennzeichnet, daß die Aktivierung folgende Schritte umfaßt:

- (a) Behandlung des Materials mit einer Lösung, die die Bindung von mindestens einer chemisch reaktiven Gruppe in dem Material ermöglicht, und
 (b) Kontaktierung des behandelten Materials mit einer Lösung, die ein bifunktionelles Vernetzungsreagens enthält, über einen ausreichend langen Zeitraum, um die Bindung der chemisch reaktiven Gruppe an das Reagens zu ermöglichen.
11. Verfahren gemäß Anspruch 10, dadurch gekennzeichnet, daß die Behandlung außerdem die Behandlung des Materials mit einer Lösung umfaßt, die die Bindung von mindestens einer chemisch aktiven Gruppe in dem Material ermöglicht, wobei die chemisch aktive Gruppe eine Carbonsäuregruppe ist.
12. Verfahren gemäß Anspruch 6, das zusätzlich den vorbereitenden Schritt der Kontaktierung des Materials mit einer Lösung umfaßt, die Verunreinigungen vom Material entfernt, wobei dieser vorbereitende Schritt vor dem Verfahrensschritt des Anbringens durchgeführt wird.
13. Verfahren gemäß Anspruch 6, dadurch gekennzeichnet, daß die Immobilisierung außerdem folgende Schritte umfaßt:
- (a) Kontaktierung des Thrombeninhibitors mit mindestens einem Molekül eines bifunktionellen Vernetzungsreagens über einen ausreichend langen Zeitraum, um die Bindung des Reagens an den Thrombeninhibitor zu ermöglichen, und
 (b) Bindung des an den Thrombeninhibitor gebundenen Reagens an die Grundsicht, wobei der gebundene Thrombeninhibitor in der Lage ist, die Thrombenbildung zu inhibieren.
14. Verfahren gemäß Anspruch 13, dadurch gekennzeichnet, daß die Kontaktierung außerdem die Kontaktierung der Grundsicht mit mindestens einem Molekül des bifunktionellen Vernetzungsreagens über einen ausreichend langen Zeitraum umfaßt, der die Bindung des Reagens an die Grundsicht ermöglicht, und die Bindung außerdem die Bindung des an den Thrombeninhibitor gebundenen Reagens an das an die Grundsicht gebundene Reagens umfaßt.
15. Verfahren gemäß Anspruch 14, das außerdem folgende Schritte umfaßt:
- (a) Reduktion des an die Grundsicht gebundenen Reagens, um darauf eine Sulfhydrylgruppe freizulegen,
 (b) Versetzen des an den Inhibitor gebundenen Reagens mit der darauf freigelegten Sulfhydrylgruppe und
 (c) Auslösen einer Substitutionsreaktion der Sulfhydrylgruppe und des an den Inhibitor gebundenen Reagens, die zur Bindung der Grundsicht an den Inhibitor führt.
16. Verfahren gemäß einem der Ansprüche 13, 14 oder 15, dadurch gekennzeichnet, daß das bifunktionelle Vernetzungsreagens aus der Gruppe heterobifunktioneller Vernetzungsreagenzien (z. B. SPDP), homobifunktioneller Vernetzungsreagenzien sowie deren Gemischen ausgewählt ist.
17. Verfahren gemäß Anspruch 13, bei dem außerdem in einem zusätzlichen Schritt das an den Thrombeninhibitor gebundene Reagens einem chromatographischen Verfahren zur Entfernung von darin enthaltenen Verunreinigungen unterzogen wird, wobei dieser zusätzliche Schritt nach der Kontaktierung und vor der Bindung durchgeführt wird.
18. Verfahren zur Herstellung einer bioverträglichen, thromboresistenten Substanz in folgenden Schritten:
- (a) Immobilisierung eines Thrombeninhibitors auf einer Grundsicht durch Verwendung eines bifunktionellen Vernetzungsreagens, z. B. eines heterobifunktionellen oder homobifunktionellen Vernetzungsreagens, wobei der Inhibitor Hirudin oder ein aktives Analog oder Fragment davon ist und die Thrombenbildung inhibiert, wenn er immobilisiert ist, und die Grundsicht eine Komponente enthält, die in der Lage ist, den Thrombeninhibitor zu binden, und
 (b) Anbringen der an den Thrombeninhibitor gebundenen Grundsicht auf mindestens einer Fläche eines synthetischen, bioverträglichen Materials.

Patentansprüche für folgende Vertragsstaaten : ES, GR

1. Verfahren zur Herstellung einer bioverträglichen, thromboresistenten Substanz, das aus folgenden Schritten besteht:
 - 5 (a) Anbringen von mindestens einer Grundsicht auf mindestens eine Fläche eines synthetischen, bioverträglichen Materials, wobei die Grundsicht eine Komponente enthält, die in der Lage ist, einen Thrombininhibitor zu binden, und
 - (b) Immobilisierung des Thrombininhibitors auf der Grundsicht durch Verwendung eines bifunktio-
10 nellen Vernetzungsreagens, z. B. eines heterobifunktionellen oder homobifunktionellen Vernetzungs-
reagens, wobei der Inhibitor Hirudin oder ein aktives Analog oder Fragment davon ist und in der Lage ist, die Thrombenbildung zu inhibieren, wenn er immobilisiert ist.
2. Verfahren gemäß Anspruch 1, dadurch gekennzeichnet, daß das Anbringen folgende Schritte umfaßt:
 - 15 (a) Aktivierung des Materials, um die Bindung der Grundsicht an das Material zu erhöhen, und
 - (b) Kontaktierung des aktivierten Materials mit der Grundsicht über einen ausreichend langen Zeitraum, um die Komponente der Grundsicht an das aktivierte Material zu binden.
3. Verfahren gemäß Anspruch 2, dadurch gekennzeichnet, daß die Aktivierung folgende Schritte umfaßt:
 - 20 (a) Behandlung des Materials mit einer Lösung, die die Bindung von mindestens einer chemisch reaktiven Gruppe in dem Material ermöglicht, und
 - (b) Kontaktierung des behandelten Materials mit einer Lösung, die ein bifunktionelles Vernetzungs-
reagens enthält, über einen ausreichend langen Zeitraum, um die chemisch reaktive Gruppe an das
Reagens zu binden.
- 25 4. Verfahren gemäß Anspruch 3, dadurch gekennzeichnet, daß die Behandlung außerdem die Behandlung des Materials mit einer Lösung umfaßt, die die Bindung von mindestens einer chemisch aktiven Gruppe in dem Material ermöglicht, wobei die chemisch aktive Gruppe eine Carbonsäuregruppe ist.
5. Verfahren gemäß Anspruch 1, das zusätzlich den vorbereitenden Schritt der Kontaktierung des
30 Materials mit einer Lösung umfaßt, die Verunreinigungen darauf entfernt, wobei dieser vorbereitende Schritt vor dem Verfahrensschritt des Anbringens durchgeführt wird.
6. Verfahren gemäß Anspruch 1, dadurch gekennzeichnet, daß die Immobilisierung außerdem folgende
35 Schritte umfaßt:
 - (a) Kontaktierung des Thrombininhibitors mit mindestens einem Molekül eines bifunktionellen
Vernetzungsreagens über einen ausreichend langen Zeitraum, um die Bindung des Reagens an den
Thrombininhibitor zu ermöglichen, und
 - (b) Bindung des an den Thrombininhibitor gebundenen Reagens an die Grundsicht, wobei der
gebundene Thrombininhibitor in der Lage ist, die Thrombenbildung zu inhibieren.
- 40 7. Verfahren gemäß Anspruch 6, dadurch gekennzeichnet, daß die Kontaktierung außerdem die Kontaktie-
rung der Grundsicht mit mindestens einem Molekül des bifunktionellen Vernetzungsreagens über
einen Zeitraum umfaßt, der lang genug ist, um die Bindung des Reagens an die Grundsicht zu
ermöglichen, und
45 die Bindung außerdem die Bindung des an den Thrombininhibitor gebundenen Reagens an das an die
Grundsicht gebundene Reagens umfaßt.
8. Verfahren gemäß Anspruch 7, das außerdem folgende Schritte umfaßt:
 - 50 (a) Reduktion des an die Grundsicht gebundenen Reagens, um darauf eine Sulfhydrylgruppe
freizulegen,
 - (b) Versetzen des an den Inhibitor gebundenen Reagens mit der darauf freigelegten Sulfhydrylgrup-
pe und
 - (c) Auslösen einer Substitutionsreaktion der Sulfhydrylgruppe und des an den Inhibitor gebundenen
Reagens, die zur Bindung der Grundsicht an den Inhibitor führt.
- 55 9. Verfahren gemäß einem der Ansprüche 6, 7 oder 8, dadurch gekennzeichnet, daß das bifunktionelle
Vernetzungsreagens aus der Gruppe heterobifunktioneller Vernetzungsreagenzien (z. B. SPDP), homo-
bifunktioneller Vernetzungsreagenzien sowie deren Gemischen ausgewählt ist.

10. Verfahren gemäß Anspruch 6, bei dem außerdem in einem zusätzlichen Schritt das an den Thrombeninhibitor gebundene Reagens einem chromatographischen Verfahren zur Entfernung von darin enthaltenen Verunreinigungen unterzogen wird, wobei dieser zusätzliche Schritt nach der Kontaktierung und vor der Bindung durchgeführt wird.

11. Verfahren zur Herstellung einer bioverträglichen, thromboresistenten Substanz in folgenden Schritten:
 (a) Immobilisierung eines Thrombeninhibitors auf einer Grundsicht durch Verwendung eines bifunktionellen Vernetzungsreagens, z. B. eines heterobifunktionellen oder homobifunktionellen Vernetzungsreagens, wobei
 der Inhibitor Hirudin oder ein aktives Analog oder Fragment davon ist und in der Lage ist, die Thrombenbildung zu inhibieren, wenn er immobilisiert ist, und
 die Grundsicht eine Komponente enthält, die in der Lage ist, einen Thrombeninhibitor zu binden, und
 (b) Anbringen der an den Thrombeninhibitor gebundenen Grundsicht auf mindestens einer Fläche eines synthetischen, bioverträglichen Materials.

12. Verfahren gemäß einem der Ansprüche 1 bis 11, dadurch gekennzeichnet, daß das Material ein Polymer enthält oder daraus besteht, z. B. ein Polymer aus der Gruppe bestehend aus Polyethylenterephthalat (z. B. Dacron), Nylon, Polyurethan, vernetztem Collagen, Polyglykolsäure, Polytetrafluorethylen sowie deren Gemischen.

13. Verfahren gemäß einem der Ansprüche 1 bis 12, dadurch gekennzeichnet, daß die Grundsicht eine Komponente enthält, die aus der Gruppe bestehend aus Protein, Peptid, Lipoprotein, Glykoprotein, Glycosaminoglycan, Hydrogel, synthetischem Polymer sowie deren Gemischen ausgewählt ist.

14. Verfahren gemäß Anspruch 13, dadurch gekennzeichnet, daß die Komponente der Grundsicht ein Protein enthält oder daraus besteht, z. B. ein Protein aus der Gruppe bestehend aus Serumalbumin (z. B. Rinderserumalbumin und Humanserumalbumin), Fibronectin (z. B. Rinderfibronectin und Humanfibronectin) sowie deren Gemischen.

Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Une substance biocompatible, thromborésistante, comprenant:

- (a) un matériau synthétique, biocompatible;
 - (b) au moins une couche basale de revêtement biocompatible ayant adhéree à au moins une surface dudit matériau; et
 - (c) un inhibiteur de la thrombogénèse immobilisé sur ladite couche basale de revêtement grâce à l'utilisation d'un réactif de réticulation bifonctionnel, par exemple un réactif de réticulation hétéro-bifonctionnel ou homo-bifonctionnel, ledit inhibiteur étant l'hirudine, ou un analogue ou un fragment actif de celle-ci,
- ladite couche basale de revêtement ayant un composant capable de lier ledit inhibiteur de la thrombogénèse, et
- ledit inhibiteur ayant une activité inhibitrice de la thrombogénèse, quand il est immobilisé.

2. La substance de la revendication 1, dans laquelle ledit matériau comprend un polymère, par exemple un polymère sélectionné dans le groupe consistant en polytéraphthalate d'éthylène (p.ex. Dacron), nylon, polyuréthane, collagène réticulé, acide polyglycolique, polytétrafluoroéthylène et en des mélanges de ceux-ci.

3. La substance de la revendication 1 ou de la revendication 2, dans laquelle ladite couche basale de revêtement comprend un composant sélectionné dans le groupe consistant en protéine, peptide, lipoprotéine, glycoprotéine, glyco-aminoglycane, hydrogel, polymère synthétique et en des mélanges de ceux-ci.

4. La substance de la revendication 3, dans laquelle ledit composant de ladite couche basale de revêtement comprend une protéine; par exemple, une protéine sélectionnée dans le groupe consistant en sérum-albumine (p.ex. sérum-albumine bovine et sérum-albumine humaine), fibronectine (p.ex.

fibronectine bovine et fibronectine humaine) et en des mélanges de celles-ci.

5. La substance de la revendication 1, dans laquelle le réactif de réticulation bifonctionnel est SPDP.
- 5 6. Une méthode de production d'une substance biocompatible, thromborésistante, ladite méthode comprenant les étapes de:
 - (a) faire adhérer au moins une couche basale de revêtement à au moins une surface d'un matériau synthétique, biocompatible, ladite couche basale de revêtement contenant un composant capable de lier un inhibiteur de la thrombogénèse; et
 - 10 (b) immobiliser ledit inhibiteur de la thrombogénèse sur ladite couche basale de revêtement grâce à l'utilisation d'un réactif de réticulation bifonctionnel, par exemple un réactif de réticulation hétéro-bifonctionnel ou homo-bifonctionnel, ledit inhibiteur étant l'hirudine, ou un analogue ou un fragment actif de celle-ci, et ayant une activité inhibitrice de la thrombogénèse, quand il est immobilisé.
- 15 7. La méthode de la revendication 6, dans laquelle ladite étape d'adhérence comprend:
 - (a) l'activation dudit matériau, de façon à augmenter la liaison de ladite couche basale de revêtement à ce dernier; et
 - (b) la mise en contact dudit matériau activé et de ladite couche basale de revêtement pendant une durée suffisante pour permettre audit composant de ladite couche basale de revêtement de se lier
 - 20 audit matériau activé.
8. La méthode de la revendication 6, dans laquelle ladite étape d'adhérence comprend l'adhérence d'une couche basale de revêtement à au moins une surface dudit matériau, ladite couche basale de revêtement contenant un composant sélectionné dans le groupe consistant en protéine, peptide,
- 25 lipoprotéine, glycoprotéine, hydrogel, glyco-aminoglycane, polymère synthétique et en des mélanges de ceux-ci.
9. La méthode de la revendication 8, dans laquelle ladite étape d'adhérence comprend, en outre, l'adhérence, à au moins une surface dudit matériau, d'une couche basale de revêtement contenant une
- 30 protéine, par exemple une protéine sélectionnée dans le groupe consistant en sérum-albumine (p.ex. sérum-albumine humaine et sérum-albumine bovine), fibronectine (p.ex. fibronectine humaine et fibronectine bovine) et en des mélanges de celles-ci.
10. La méthode de la revendication 7, dans laquelle ladite étape d'activation comprend les étapes de:
 - (a) traiter ledit matériau avec une solution qui rend utilisable pour la liaison, au moins un groupe chimiquement réactif dudit matériau; et
 - (b) mettre en contact ledit matériau traité avec une solution contenant un réactif de réticulation bifonctionnel pendant une durée suffisante pour permettre la liaison dudit groupe chimiquement réactif audit réactif.
- 40 11. La méthode de la revendication 10, dans laquelle ladite étape de traitement comprend, en outre, le traitement dudit matériau avec une solution qui rend utilisable pour la liaison, au moins un groupe chimiquement actif dudit matériau, ledit groupe chimiquement actif étant un groupe acide carboxylique.
- 45 12. La méthode de la revendication 6 comprenant, en outre, l'étape préliminaire de mettre en contact ledit matériau avec une solution qui élimine les impuretés de ce dernier, ladite étape préliminaire étant accomplie avant ladite étape d'adhérence.
13. La méthode de la revendication 6, dans laquelle ladite étape d'immobilisation comprend, en outre, les
- 50 étapes de:
 - (a) mettre en contact ledit inhibiteur de la thrombogénèse avec au moins une molécule d'un réactif de réticulation bifonctionnel, pendant une durée suffisante pour permettre la liaison dudit réactif audit inhibiteur de la thrombogénèse; et
 - (b) lier ledit réactif lié à l'inhibiteur de la thrombogénèse audit revêtement basal, ledit inhibiteur de la
 - 55 thrombogénèse lié ayant une activité inhibitrice de la thrombogénèse.
14. La méthode de la revendication 13, dans laquelle ladite étape de mise en contact comprend, en outre, la mise en contact dudit revêtement basal avec au moins une molécule dudit réactif de réticulation

bifonctionnel pendant une durée suffisante pour permettre la liaison dudit réactif audit revêtement basal,

et ladite étape de liaison comprend, en outre, la liaison dudit réactif lié à l'inhibiteur de la thrombogénèse audit réactif lié au revêtement basal.

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15. La méthode de la revendication 14 comprenant, en outre, les étapes de:

- (a) réduire ledit réactif lié au revêtement basal, pour exposer un groupe sulfhydryle sur celui-ci;
- (b) ajouter ledit réactif lié à l'inhibiteur au groupe sulfhydryle exposé; et
- (c) induire une réaction de substitution impliquant ledit groupe sulfhydryle et ledit réactif lié à l'inhibiteur, ladite réaction résultant en une liaison dudit revêtement basal audit inhibiteur.

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16. La méthode de l'une quelconque des revendications 13, 14 ou 15, dans laquelle ledit réactif de réticulation bifonctionnel est sélectionné dans le groupe consistant en réactifs de réticulation hétéro-bifonctionnels (par exemple SPDP), réactifs de réticulation homo-bifonctionnels et en des mélanges de ceux-ci.

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17. La méthode de la revendication 13 comprenant, en outre, l'étape additionnelle de soumettre ledit réactif lié à l'inhibiteur de la thrombogénèse à une procédure chromatographique, pour éliminer les impuretés de ce dernier, ladite étape additionnelle étant accomplie après ladite étape de mise en contact et avant ladite étape de liaison.

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18. Une méthode de production d'une substance biocompatible, thromborésistante, ladite méthode comprenant les étapes de:

- (a) immobiliser un inhibiteur de la thrombogénèse sur une couche basale de revêtement grâce à l'utilisation d'un réactif de réticulation bifonctionnel, par exemple un réactif de réticulation hétéro-bifonctionnel ou homo-bifonctionnel,

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ledit inhibiteur étant l'hirudine, ou un analogue ou un fragment actif de celle-ci, et ayant une activité inhibitrice de la thrombogénèse, quand il est immobilisé, et

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ladite couche basale de revêtement contenant un composant capable de lier ledit inhibiteur de la thrombogénèse; et

- (b) faire adhérer ladite couche basale de revêtement liée audit inhibiteur de la thrombogénèse à au moins une surface d'un matériau synthétique, biocompatible.

Revendications pour les Etats contractants suivants : ES, GR

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1. Une méthode de production d'une substance biocompatible, thromborésistante, ladite méthode comprenant les étapes de:

- (a) faire adhérer au moins une couche basale de revêtement à au moins une surface d'un matériau synthétique, biocompatible, ladite couche basale de revêtement contenant un composant capable de lier un inhibiteur de la thrombogénèse; et

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(b) immobiliser ledit inhibiteur de la thrombogénèse sur ladite couche basale de revêtement grâce à l'utilisation d'un réactif de réticulation bifonctionnel, par exemple un réactif de réticulation hétéro-bifonctionnel ou homo-bifonctionnel, ledit inhibiteur étant l'hirudine, ou un analogue ou un fragment actif de celle-ci, et ayant une activité inhibitrice de la thrombogénèse, quand il est immobilisé.

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2. La méthode de la revendication 1, dans laquelle ladite étape d'adhérence comprend:

- (a) l'activation dudit matériau, de façon à augmenter la liaison de ladite couche basale de revêtement à ce dernier; et

(b) la mise en contact dudit matériau activé et de ladite couche basale de revêtement pendant une durée suffisante pour permettre audit composant de ladite couche basale de revêtement de se lier audit matériau activé.

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3. La méthode de la revendication 2, dans laquelle ladite étape d'activation comprend les étapes de:

- (a) traiter ledit matériau avec une solution qui rend utilisable pour la liaison, au moins un groupe chimiquement réactif dudit matériau; et

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(b) mettre en contact ledit matériau traité avec une solution contenant un réactif de réticulation bifonctionnel pendant une durée suffisante pour permettre la liaison dudit groupe chimiquement réactif audit réactif.

4. La méthode de la revendication 3, dans laquelle ladite étape de traitement comprend, en outre, le traitement dudit matériau avec une solution qui rend utilisable pour la liaison, au moins un groupe chimiquement actif dudit matériau, ledit groupe chimiquement actif étant un groupe acide carboxylique.
5. La méthode de la revendication 1 comprenant, en outre, l'étape préliminaire de mettre en contact ledit matériau avec une solution qui élimine les impuretés de ce dernier, ladite étape préliminaire étant accomplie avant ladite étape d'adhérence.
6. La méthode de la revendication 1, dans laquelle ladite étape d'immobilisation comprend, en outre, les étapes de:
 - (a) mettre en contact ledit inhibiteur de la thrombogénèse avec au moins une molécule d'un réactif de réticulation bifonctionnel, pendant une durée suffisante pour permettre la liaison dudit réactif audit inhibiteur de la thrombogénèse; et
 - (b) lier ledit réactif lié à l'inhibiteur de la thrombogénèse audit revêtement basal, ledit inhibiteur de la thrombogénèse lié ayant une activité inhibitrice de la thrombogénèse.
7. La méthode de la revendication 6, dans laquelle ladite étape de mise en contact comprend, en outre, la mise en contact dudit revêtement basal avec au moins une molécule dudit réactif de réticulation bifonctionnel pendant une durée suffisante pour permettre la liaison dudit réactif audit revêtement basal,
 - et ladite étape de liaison comprend, en outre, la liaison dudit réactif lié à l'inhibiteur de la thrombogénèse audit réactif lié au revêtement basal.
8. La méthode de la revendication 7 comprenant, en outre, les étapes de:
 - (a) réduire ledit réactif lié ou revêtement basal, pour exposer un groupe sulfhydryle, sur celui-ci;
 - (b) ajouter ledit réactif lié à l'inhibiteur au groupe sulfhydryle exposé; et
 - (c) induire une réaction de substitution impliquant ledit groupe sulfhydryle et ledit réactif lié à l'inhibiteur, ladite réaction résultant en une liaison dudit revêtement basal audit inhibiteur.
9. La méthode de l'une quelconque des revendications 6, 7 ou 8, dans laquelle ledit réactif de réticulation bifonctionnel est sélectionné dans le groupe consistant en réactifs de réticulation hétéro-bifonctionnels (par exemple SPDP), réactifs de réticulation homobifonctionnels et en des mélanges de ceux-ci.
10. La méthode de la revendication 6 comprenant, en outre, l'étape additionnelle de soumettre ledit réactif lié à l'inhibiteur de la thrombogénèse à une procédure chromatographique, pour éliminer les impuretés de ce dernier, ladite étape additionnelle étant accomplie après ladite étape de mise en contact et avant ladite étape de liaison.
11. Une méthode de production d'une substance biocompatible, thromborésistante, ladite méthode comprenant les étapes de:
 - (a) immobiliser un inhibiteur de la thrombogénèse sur une couche basale de revêtement grâce à l'utilisation d'un réactif de réticulation bifonctionnel, par exemple un réactif de réticulation hétéro-bifonctionnel ou homo-bifonctionnel,
 - ledit inhibiteur étant l'hirudine, ou un analogue ou un fragment actif de celle-ci, et ayant une activité inhibitrice de la thrombogénèse, quand il est immobilisé, et
 - ladite couche basale de revêtement contenant un composant capable de lier ledit inhibiteur de la thrombogénèse; et
 - (b) faire adhérer ladite couche basale de revêtement liée audit inhibiteur de la thrombogénèse à au moins une surface d'un matériau synthétique, biocompatible.
12. La méthode de l'une quelconque des revendications 1 à 11, dans laquelle ledit matériau comprend un polymère, par exemple, un polymère sélectionné dans le groupe consistant en polytéraphthalate d'éthylène (p.ex. Dacron), nylon, polyuréthane, collagène réticulé, acide polyglycolique, polytétrafluoroéthylène et en des mélanges de ceux-ci.
13. La méthode de l'une quelconque des revendications 1 à 12, dans laquelle ladite couche basale de revêtement comprend un composant sélectionné dans le groupe consistant en protéine, peptide, lipoprotéine, glycoprotéine, glyco-aminoglycane, hydrogel, polymère synthétique et en des mélanges

de ceux-ci.

14. La méthode de la revendication 13, dans laquelle ledit composant de ladite couche basale de revêtement comprend une protéine; par exemple, une protéine sélectionnée dans le groupe consistant en sérum-albumine (p.ex. sérum-albumine bovine et sérum-albumine humaine), fibronectine (p.ex. fibronectine bovine et fibronectine humaine) et en des mélanges de celles-ci.

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FIGURE 1

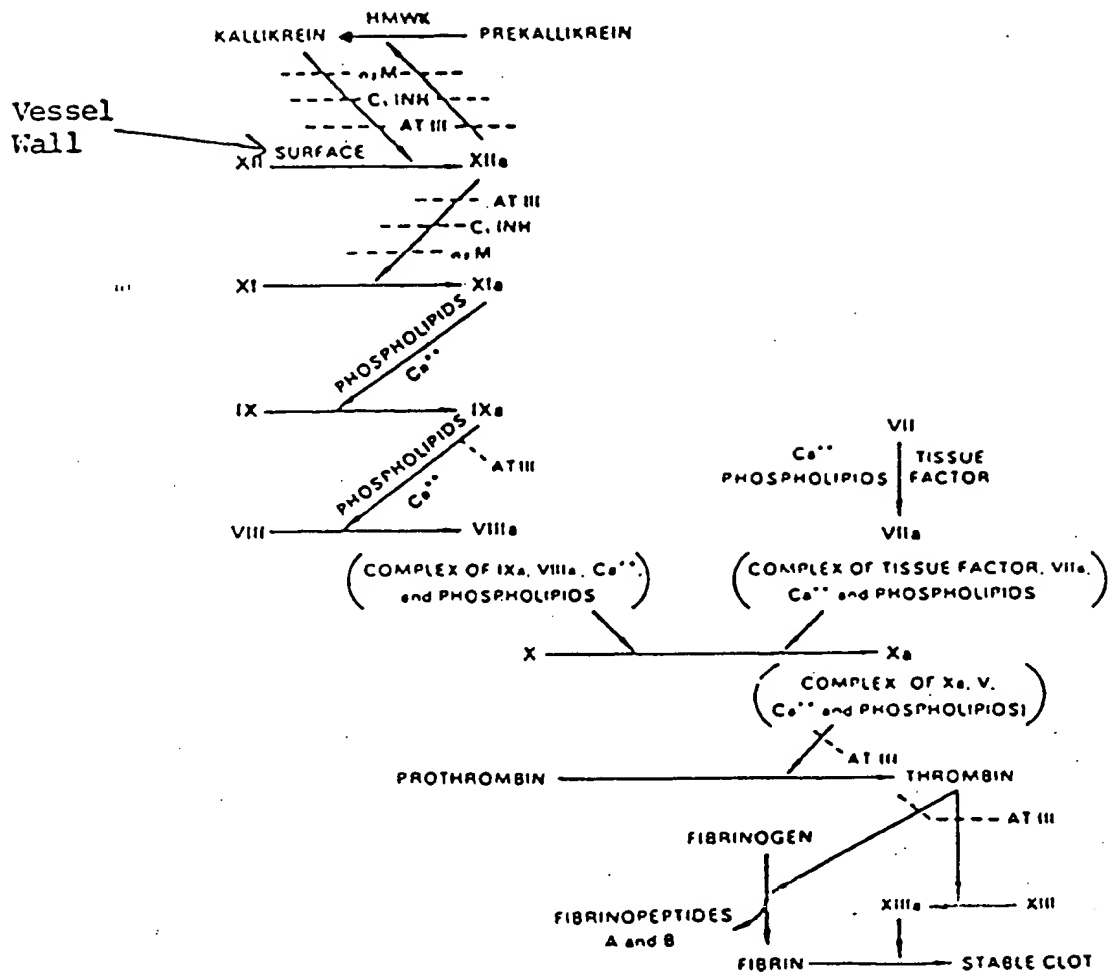


FIGURE 2

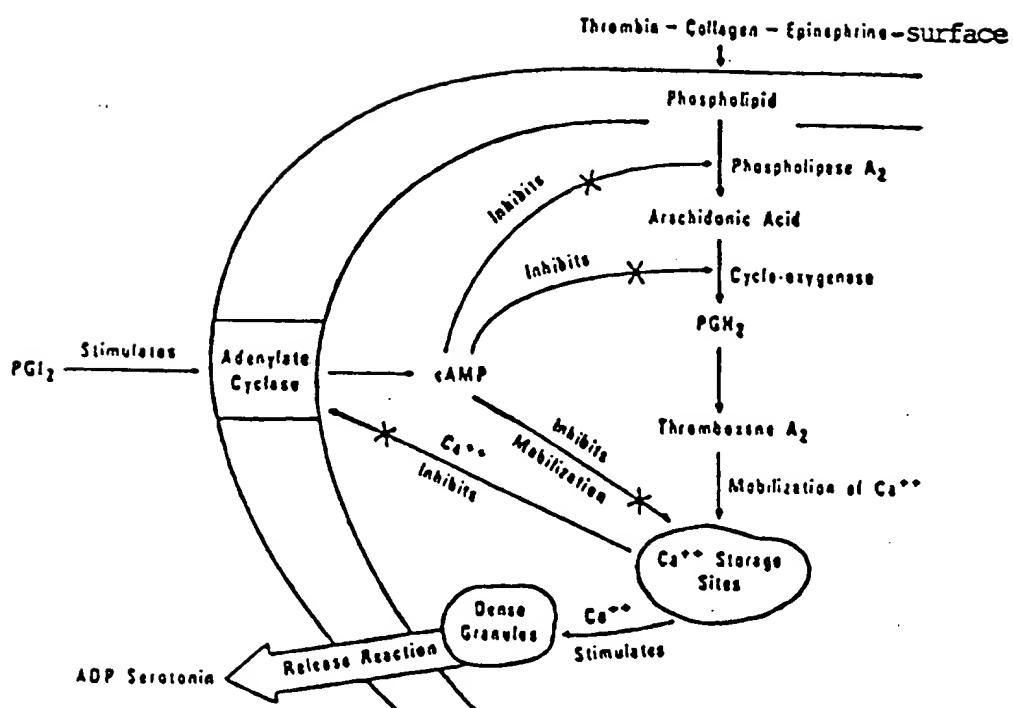


FIGURE 3

10 (A)

H--Val--Val--Tyr--Thr--Asp--Cys--Thr--Glu--Ser--Gly--

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--Gln--Asn--Leu--Cys--Leu--Cys--Glu--Gly--Ser--Asn--

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--Val--Cys--Gly--Gln--Gly--Asn--Lys--Cys--Ile--Leu--

40

--Gly--Ser--Asp--Gly--Glu--Lys--Asn--Gln--Cys--Val--

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--Thr--Gly--Glu--Gly--Thr--Pro--Lys--Pro--Gln--Ser--

60

--His--Asn--Asp--Gly--Asp--Phe--Glu--Glu--Ile--Pro--

SO₃H

--Glu--Glu--Tyr--Leu--Gln--OH

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